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Cyclosporine enhances the expression of TGF-β in the juxtaglomerular cells of the rat kidney

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Cyclosporine enhances the expression of TGF-β in the juxtaglomerular cells of the rat kidney. The mediators of cyclosporine (CsA) nephrotoxicity remain ill defined. In this study, we describe evidence of increased amounts of transforming growth factor-beta (TGF-β) in the kidneys of adult male Wistar rats treated with CsA (5 to 25 mg/kg/day) for four weeks. Localization of TGF-β was undertaken immunocytochemically at both light and electron microscope levels and Northern blot analysis was applied to detect changes in transcription of TGF-β. In control rats, weak to moderate immunostaining for TGF-β was observed, in the juxtaglomerular arterioles. CsA treatment resulted in a dose-dependent increase in the number of stained afferent and interlobular arterioles and in the intensity of staining. The number of stained afferent arterioles increased from a control value of 0.21 ± 0.08/mm^2 cortex to 0.84 ± 0.15/mm^2 cortex, $P < 0.01$, and to 1.12 ± 0.10/mm^2 cortex, $P < 0.01$, in rats treated with CsA 12.5 mg/kg/day and 25 mg/kg/day, respectively. The number of interlobular arterioles stained for TGF-β increased from a control value of 0.07 ± 0.05/mm^2 to 0.31 ± 0.02/mm^2, $P < 0.05$, and 0.39 ± 0.07/mm^2, $P < 0.01$, in rats treated with CsA, 12.5 mg/kg/day and 25 mg/kg/day, respectively. At the electron microscope level, TGF-β was localized exclusively within the granular cells of the juxtaglomerular arterioles. Northern blot analysis suggested that this enhanced staining is due to increased transcription of TGF-β1. We have therefore observed an association between TGF-β and CsA-induced nephrotoxicity. While this does not establish a causal link, it leads us to postulate that TGF-β, alone or in combination with other growth factors, may play a role in the pathogenesis of CsA induced nephrotoxicity.

There is no doubt that the success of solid organ transplantation over the last decade is due to the introduction of cyclosporine (CsA) [1–3]. Despite this improvement in allograft and patient survival, the use of CsA is tempered by its nephrotoxic side effects [4–6]. CsA nephrotoxicity can be identified by acute functional effects and chronic histological changes. Functional changes include increased renal vascular resistance and decreased renal plasma flow and glomerular filtration [7–9]. Chronic structural changes are marked by arteriolopathy, tubular atrophy and interstitial fibrosis [10]. The mechanism and mediators of these hemodynamic and structural changes are ill defined, and possible links between functional changes and structural damage remain to be elucidated.

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A wide range of mediators have been implicated in the acute hemodynamic effects of CsA, including angiotensin II [7, 11, 12], an activated sympathetic nervous system [7, 11, 13], thromboxane A_2 [14–16], endothelin [17–19], and most recently, changes in the nitric oxide system [20–22].

We have recently reported the presence of immunoreactive platelet-derived growth factor (PDGF) in the renal cortical arterioles of rats injected with nephrotoxic doses of CsA and suggested that PDGF, with its inherent vasoconstrictive and fibrogenic effects may be involved in the pathogenesis of CsA-induced nephrotoxicity [23]. Furthermore, the ultrastructural localization of PDGF within hypertrophied juxtaglomerular cells suggest that it may also mediate the hypertrophy of the juxtaglomerular cells which is seen in animals treated with nephrotoxic doses of CsA [24].

Transforming growth factor-beta (TGF-β) has been shown to be increased in other experimental models of juxtaglomerular cell hypertrophy, including that caused by water deprivation [25] and potassium depletion in rodents [26], and in infants with renal ischemia [27]. These findings, together with the known mitogenic effect of TGF-β on vascular smooth muscle cells, have led to the hypothesis that TGF-β is involved in hypertrophy of the juxtaglomerular cells observed in these conditions.

The observations described above and the frequent co-localization of PDGF and TGF-β in mesenchymal cells have prompted us to investigate the changes in juxtaglomerular TGF-β in the kidneys of rats treated with CsA.

Methods

Animals

Adult male Wistar rats (Sheffield University strain) weighing 250 to 300 g at the outset of the experiments were housed two to three to a cage and maintained at 20°C and 45% humidity on a 12-hour light/dark cycle. They were allowed free access to standard rat chow (Labsure, March, Cambridge, UK) and water, except one group of pair-fed animals (see below). All procedures were carried out under licence according to regulations laid down by Her Majesty's Government, United Kingdom (Animals Scientific Procedures Act, 1986).

Drug preparation

Cyclosporine (CsA) was a gift of Sandoz UK Ltd. (Cambridge, UK). It was dissolved first in ethanol (1 g/ml) and then diluted

with olive oil (low acidity, Sigma, Poole, UK) to a concentration of 10 mg/ml.

Experimental protocols

Rats were injected intraperitoneally with 5, 7.5, 10 mg CsA/kg body wt ($N = 3$ per dose), 12.5 or 25 mg CsA/kg body wt ($N = 6$ per dose), daily for four weeks. Control animals ($N = 3$ to 6) were injected with an equivalent volume of the olive oil vehicle. The food and water consumption of these rats was measured over periods of 24 hours, at weekly intervals.

To test whether the observed changes were secondary to loss of appetite and/or dehydration, a group of rats ($N = 6$) was injected with 25 mg/kg/day CsA for four weeks, and their daily food and water consumption was carefully measured. These rats were placed individually in wire bottomed cages and the average daily food and water consumption of each rat was measured. The average of two consecutive days consumption was then given to the pair fed and watered control rat ($N = 6$), two days in arrears.

To establish whether the effects of CsA were due to ischemia, a further group of control rats ($N = 6$) underwent partial ligation of the aorta. For this, rats were anaesthetized and the left kidney and aorta were exposed through a lumbar incision. A silk suture was then passed around the aorta between the origins of the left and right renal arteries and tied over a needle stylet of 0.1 mm external diameter [28]. This resulted in a reproducible reduction in blood flow to the left kidney. These rats were killed after two weeks.

At the start of the experiment and at sacrifice, all rats were weighed and placed individually in metabolic cages for 48 hours. After 24 hours of acclimation, urine was collected over a 24 hour period for the determination of creatinine clearance rates. Also, blood samples were withdrawn from the tail vein for the determination of serum creatinine, potassium and urea concentrations (standard autoanalyser techniques, Monarch Instruments, UK Ltd.). Blood levels of CsA were also determined by radioimmunoassay [29]. Systolic blood pressure was measured by the tail cuff method while the rats were lightly anaesthetized with ether.

Tissue preparation

Before sacrifice, the rats were deeply anaesthetized with Sagatal (Pentobarbitone sodium, RMB Animal Health, Dagenham, UK), weighed and samples of blood were taken. The kidneys were then perfused with phosphate buffered saline (PBS) pH 7.2 via a cannula inserted into the left ventricle of the heart with blood allowed to drain via a small incision made in the right atrium. When perfused, the left kidney was cut into 2 to 3 mm thick slices, weighed and snap frozen with liquid nitrogen for RNA analyses. The right kidney was processed for light and electron microscope immunohistochemistry.

For light microscope immunohistochemistry, tissue was fixed overnight in 4% formol-calcium, dehydrated with alcohol, cleared with chloroform and embedded in low melting point paraffin wax (56°C).

For electron microscope immunocytochemistry, tissue was fixed in 4% wt:vol paraformaldehyde, dehydrated to 50% ethanol at 4°C and then transferred to a deep freeze cabinet at −30°C where dehydration was completed. Tissues were then embedded and polymerized at low temperature (−30°C) in Lowicryl HM20 resin (Agar Scientific, Standstead, UK) as previously described [24].

Light microscope histology

Dewaxed paraffin embedded specimens were routinely stained with hematoxylin and eosin (H&E), with Masson's trichrome stain and by the periodic acid-Schiff (PAS) procedure.

Light microscope immunohistochemistry

Sections 4 μm thick were mounted on gelatinized glass slides and stained immunohistochemically by an avidin-biotinylated horseradish peroxidase procedure, using a commercially available kit (ABC Elite Kit, Vector Laboratories, Peterborough, UK) as previously described [23]. Briefly, sections were dewaxed with xylene and hydrated in graded water:ethanol solutions. Endogenous peroxidase activity was quenched by immersing sections in 0.6% H_2O_2 in methanol for 20 minutes and sections were then incubated for 30 minutes with 1.5% goat or horse serum as appropriate. Sections were then incubated overnight at 4°C in a humid atmosphere with a polyclonal rabbit anti-human TGF-β1 and 2 (R & D Systems, Abingdon, UK) or with a mouse monoclonal anti-smooth muscle actin (anti-α-SMA_1), a gift from Professor G. Gabbiani (Geneva, Switzerland), which specifically binds to vascular smooth muscle cells [30]. Antibodies were diluted with phosphate buffered saline (PBS) containing 0.01% crystalline grade bovine serum albumin (BSA, biotin free, Sigma). Sections were then incubated with the relevant secondary antibody for 30 minutes, rinsed in PBS and incubated with the peroxidase substrate, 3-amino 9-ethyl carbazole (AEC Kit, Vector). Finally, sections were counterstained with dilute aqueous hematoxylin and mounted with Glycergel (Dako, High Wycombe, UK).

Control incubations involved preadsorption of the antibody with TGF-β1 and TGF-β2 antigens (R&D Systems, UK), the substitution of the primary antibody with an equivalent concentration of rabbit or mouse immunoglobulin, as appropriate, or the omission of the primary antibody.

For evaluation the intensity of staining an arbitrary score from + to +++ was used, where + = weak, ++ = moderate, and +++ = strong staining.

Light microscope morphometric analysis

The number of juxtaglomerular arteriole profiles stained for TGF-β per mm^2 kidney cortex was compared with those stained with α-SMA_1. For this, horizontal serial sections cut through the hilus of the right kidney of each animal and adjacent sections were stained for TGF β or α-SMA_1, respectively. Sections were incubated with α-SMA_1, which facilitates the identification of glomerular and interlobular arterioles for the morphometric analysis and for comparison with the TGF-β staining. Sections were viewed down a light microscope fitted with an ×10 flat-field objective, and the total numbers of glomeruli and stained juxtaglomerular arterioles were recorded. The cortical area of each of these sections was measured using a Seescan image analyzer. The results are expressed as the mean number of glomeruli or stained juxtaglomerular arterioles per mm^2 of kidney cortex.

Electron microscope immunocytochemistry

Electron microscope immunocytochemical studies were carried only on tissue from rats treated with CsA (12.5 mg/kg/day) for four weeks. Ultrathin section (100 nm) were mounted on Collodion-coated nickel grids and incubated with TGF-β to demonstrate its ultrastructural localization, using a Protein A-gold

Table 1. Body weight gain, blood pressure, blood and serum levels of creatinine, potassium and CsA as well as creatinine clearance of control, pair-fed control and CsA-treated rats after 1 month of daily injections

	N	Body weight gain g	Serum creatinine μmol/liter	Serum potassium mmol/liter	Creatinine clearance rate ml/min/100 g body wt	Systolic blood pressure mm Hg	CsA blood level ng/ml
Control	3	104 ± 11	35 ± 5	4.9 ± 0.4	0.52 ± 0.03	118 ± 7	—
CsA, 5 mg/kg	3	91 ± 4	34 ± 4	4.7 ± 0.3	0.45 ± 0.01	109 ± 5	423 ± 89
CsA, 7.5 mg/kg	3	88 ± 16[a]	39 ± 3[a]	5.0 ± 0.3	0.46 ± 0.02	123 ± 2	497 ± 155
CsA, 10 mg/kg	3	67 ± 7[a]	45 ± 6[a]	4.9 ± 0.1	0.43 ± 0.04[a]	121 ± 3	824 ± 91
Control	6	88 ± 5	35 ± 2	5.8 ± 0.4	0.53 ± 0.01	106 ± 9	—
CsA, 12.5 mg/kg	6	59 ± 5[c]	47 ± 3[c]	4.9 ± 0.1	0.29 ± 0.01[c]	118 ± 3	806 ± 75
Control	6	101 ± 13	42 ± 4	4.8 ± 0.3	0.49 ± 0.03	111 ± 7	—
CsA, 25 mg/kg	6	42 ± 9[c]	71 ± 8[c]	4.8 ± 0.3	0.28 ± 0.04[c]	130 ± 3[a]	3875 ± 484
Pair-fed control	6	74 ± 7	48 ± 3	5.1 ± 0.3	0.39 ± 0.01	129 ± 9	—
CsA, 25 mg/kg	6	49 ± 6[c]	73 ± 7[c]	5.4 ± 0.4	0.26 ± 0.05[c]	131 ± 3[a]	2272 ± 106

Results are expressed as mean ± SEM.
Statistical analysis by one way ANOVA. Differences not significant unless stated otherwise.
[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$

labeling procedure [31]. Briefly, grids were first inverted successively on each of the following blocking solutions at room temperature: (a) PBS containing 0.1% ovalbumin and 0.1% Tween 20 (POT) for 10 minutes; (b) POT containing 0.1% wt:vol cold-water fish gelatine (Sigma) for 10 minutes; (c) POT containing 0.02 M glycine for three minutes. The grids were then incubated with anti-TGF-β diluted (1:40) with POT, overnight at 4°C. The grids were then rinsed with PBS and treated with Protein-A coupled to 10 nm colloidal gold (Amersham International, Aylesbury, UK, diluted 1:20 with POT) for two hours, at 4°C. Next, the grids were rinsed in PBS and then fixed in 1% glutaraldehyde for five minutes, at room temperature. Finally, the grids were rinsed with distilled water and briefly counterstained with uranyl acetate (5 min) and lead citrate (2 min). Sections were examined in Philips CM10 electron microscope operated at 80 KV and fitted with 25 mm objective aperture.

Control sections were treated in a similar fashion to that described for light microscopy immunohistochemistry. In addition, sections were also incubated with an inappropriate antibody (polyclonal goat anti-human collagen type IV; Southern Biotechnology, Europath, Wales, UK).

RNA analysis

Northern blot analysis was carried on tissues from rats injected with CsA (12.5 mg/kg/day).

A TGF-β1 specific Kpn, Apa-1 600 bp fragment (amino acids 68 to 268 of the precursor region) of the full length murine TGF-β1 [32] was subcloned in to pBluescribe (Stratagene, UK) [33]. The 600 bp fragment was excised using a *Hin*dIII/*Eco*RI digest.

A TGF-β2 specific Pst-1, Sac-1 500 bp fragment (amino acids 81 to 249) of the full length TGF-β2 [34] was prepared by PCR [35] and then subcloned into pBluescribe (Stratagene) [33]. The 500 bp fragment was excised using a Pst-1, Sac-1 digest. Both the TGF-β1 and TGF-β2 cDNA's were provided by Dr. R. Akhurst (Duncan Guthrie Institute of Medical Genetics, Glasgow, UK).

Excised cDNA fragments were separated by electrophoresis on a 1% (wt/vol) agarose TAE gel and then purified from the agarose using Bandprep™ (Pharmacia, UK) according to the manufacturer's instructions. Twenty-five nanograms of the purified cDNA were random primed with ^{32}P labeled dCTP (Redivue, Amersham, UK) using the Promega Random Prime System (Promega, UK). Unincorporated label was removed using Sephadex G50 Nick Columns™ (Pharmacia) according to the manufacturer's instructions. For RNA analysis, total RNA was extracted using the RNAsol B™ (Cinnia/Biotex, distributed by Biogenesis, UK) and quantified by spectrophotometry. Fifteen micrograms of total RNA were electrophoresed on a 1% agarose MOPS/formaldehyde gel and viewed under U.V. light to verify loading and the presence of intact ribosomal bands. RNA was then transferred to N^+ nylon (Boehringer Mannheim, UK) by capillary blotting with 20 × SSC. Transferred RNA was cross-linked to the nylon filter using U.V. irradiation (Stratalinker, Stratagene).

Prehybridization and hybridization were performed as described in detail elsewhere [36]. Briefly, filters were prehybridized in a mixture containing 50% (vol:vol) de-ionized formamide, 5 × SSPE, 5 × Denhardt's reagent, 1% (wt:vol) SDS and 200 mg/ml sonicated and denatured salmon sperm DNA, at 37°C for one hour. Hybridization was performed under the same conditions with the addition of labeled probe to 1×10^6 cpm/ml for 18 hours. Filters were then washed to a stringency of 0.2% SDS/0.2 × SSPE at 60°C for one hour and then exposed to Kodak XOMAT LS film for four days with intensifying screens. Developed films were subsequently analyzed densitometrically using a Seescan image analyzer. Optical density values for TGF-β were standardized by reference to the optical density of ethidium bromide stained gels of ribosomal 28 s RNA.

Statistical analysis

Results are expressed as mean ± SEM. The significance between groups was determined either by a modified Student's *t*-test, Welch test, or by an analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. In each case, a probability of less than 5% was taken as significant.

Results

General observations

CsA-treated rats gained weight at a slower rate than control animals for all doses of the drug including the pair-fed group (Table 1). There was no significant differences in water consumption between any of the groups. No change in food consumption was noted with doses of CsA from 5 mg/kg/day to 12.5 mg/kg/day inclusive. In rats treated with 25 mg/kg/day, food consumption

declined from 24 g/day/rat at the outset to 18 g/day/rat after seven days, but remained constant thereafter.

CsA-treated rats showed significantly higher serum creatinine concentrations after four weeks of treatment when doses of 7.5 mg/kg body wt/day and more were used (Table 1). However, significant reductions in creatinine clearance were only evident in those rats treated with 12.5 mg/kg/day or more (Table 1). No significant difference was observed in serum potassium concentration between CsA-treated and control rats, for any dose (Table 1). Whole blood CsA levels increased with the dose of drug and reached a concentration of 806 ± 75 ng/ml with 12.5 mg/kg/day CsA and 2272 ± 106 ng/ml with 25 mg/kg/day at the time of sacrifice (Table 1). Mean systolic blood pressure was only significantly different from controls in the group treated with the highest dose of CsA (25 mg/kg/day, Table 1). Equivalent data for the control group of ischemic animals are shown in Table 2.

Histological changes

When the lower doses of CsA were given (5 to 10 mg/kg/day) there was little evidence of histological change apart from pronounced PAS staining of some periglomerular and interlobular arterioles (Fig. 1A). With higher doses (12.5 or 25 mg/kg/day), strong PAS staining was accompanied by capsular notching and increased basophilia of the underlying tubules (Fig. 1B). Sections from rats treated with CsA 25 mg/kg/day stained with Masson's trichrome stain showed substantial amounts of collagen in the interstitium of these areas (Fig. 1C). Furthermore, some proximal tubule profiles displayed isometric vacuolation and a few others appeared either collapsed or dilated (Fig. 1D). However, in all cases the brush borders appeared intact and no tubular casts were evident.

In the ischemia group, the ischemic left kidney displayed proximal tubular vacuolation. Arteriolar hypertrophy and hyalinosis were also evident as well as an expansion of the vascular adventitia. The control right kidney was structurally normal.

Light microscope immunohistochemistry and morphometry

Incubation with the anti-TGF-β antibody resulted in the staining of the juxtaglomerular arterioles and some segments of interlobular arterioles in CsA-treated rats (Fig. 2 A, B). The size of the stained areas increased with the dose of CsA up to the level of 12.5 mg/kg/day. Control tissue also stained with this antibody but was confined to occasional juxtaglomerular arterioles containing one or two stained cells (Fig. 2C), with minimal involvement of the interlobular arterioles.

Staining intensity of tissue from control rats and those injected with CsA in either a dose of 5 mg/kg/day or 7.5 mg/kg/day was mostly (~78%) weak with some (~18%) arterioles exhibiting moderate staining (Table 2). Those rats treated with 10 mg/kg/day displayed a range of weak to strong staining, while those rats injected with CsA in doses of 12.5 or 25 mg/kg invariably exhibited the strongest staining for immunoreactive TGF-β. (Table 2). Control sections tested with a non-immune serum (Fig. 2D) or with antibody pre-adsorbed with the TGF-β1 and those incubated in the absence of primary antibody showed no specific immunostain.

Rats subjected to renal ischemia showed increased staining for TGF-β in the left (ischemic) kidney, similar to that seen with CsA 10 mg/kg/day, whereas the right kidney showed a pattern and intensity of staining similar to control olive oil-treated rats (Fig. 2 E, F).

There was no significant difference between CsA-treated and control animals in the number of afferent/efferent arterioles per mm^2 of cortex which stained with α-SMA_1 (Table 3). However, the number of profiles which stained with TGF-β did increase following CsA treatment, to 56% and 77% of periglomerular arterioles (with 12.5 mg/kg/day and 25 mg/kg/day CsA, respectively) compared with only 14% in control animals (Table 3). There was also a four- to sixfold increase in the number of interlobular arterioles which stained with TGF-β following CsA treatment (Table 3). However, the number of arterioles which stained in pair fed and watered rats was not significantly different from control values. In rats subjected to renal ischemia, there was a twofold increase in the number of stained arterioles in the left ischemic kidney compared to the right control kidney (Table 3), but there was no significant difference in the number of stained afferent or interlobular arterioles between the right control kidney and the other control groups.

Electron microscope immunocytochemistry

At the electron microscope level, incubation of ultrathin sections with anti-TGF-β followed by Protein A coupled to 10 nm colloidal gold established that TGF-β immunostain was confined to the secretory granules within the juxtaglomerular cells of the afferent arterioles (Fig. 3 A, B) and occasional efferent arterioles. The granules of transformed myocytes in the walls of interlobular arterioles of CsA-treated rats also stained. Granulated juxtaglomerular cells were encountered far less frequently in control tissue, and usually contained fewer granules. However, these granules stained for TGF-β but generally with a lower density than seen in CsA-treated tissue (Fig. 3C). No gold particles were evident over any of the adjoining structures, such as the muscle wall of the arteriole, the endothelium or the intercellular matrix. Furthermore, no gold particles were evident when the primary antibody was substituted by non-immune goat serum (Fig. 3D) nor when an inappropriate antibody was applied (Fig. 3E). A similar distribution of gold particles to that of CsA treated rats was seen in the juxtaglomerular cells of ischemic kidney (Fig. 3F).

Northern blot analysis

Northern blot analysis of total RNA samples from control olive oil-treated and CsA-treated rat kidney samples showed a single band of approximately 2.3 kD when probed with the TGF-β1 cDNA (Fig. 4), but no bands were apparent after hybridization with the TGF-β2 probe. The standardized average optical density of the TGF-β1 bands from CsA-treated samples was significantly greater than those from control tissue (O.D. 0.073 ± 0.019 vs. O.D. 0.010 ± 0.003, respectively, $P < 0.05$) when variations in gel loading were accounted for by comparison with ribosomal 28 s RNA.

Discussion

In these studies, we have demonstrated that treatment with CsA in daily doses ranging from 5 mg/kg/day to 25 mg/kg of body wt results in an increase in renal immunostaining for TGF-β. The staining was most intense at doses of 12.5 mg/kg or higher. We believe the observed immunostaining to be specific to TGF-β1 as pre-absorption of the antibody with recombinant human TGF-β1 abolished the staining. Northern blot analysis confirmed such an assertion as an increase in the amount of TGF-β1 mRNA and not

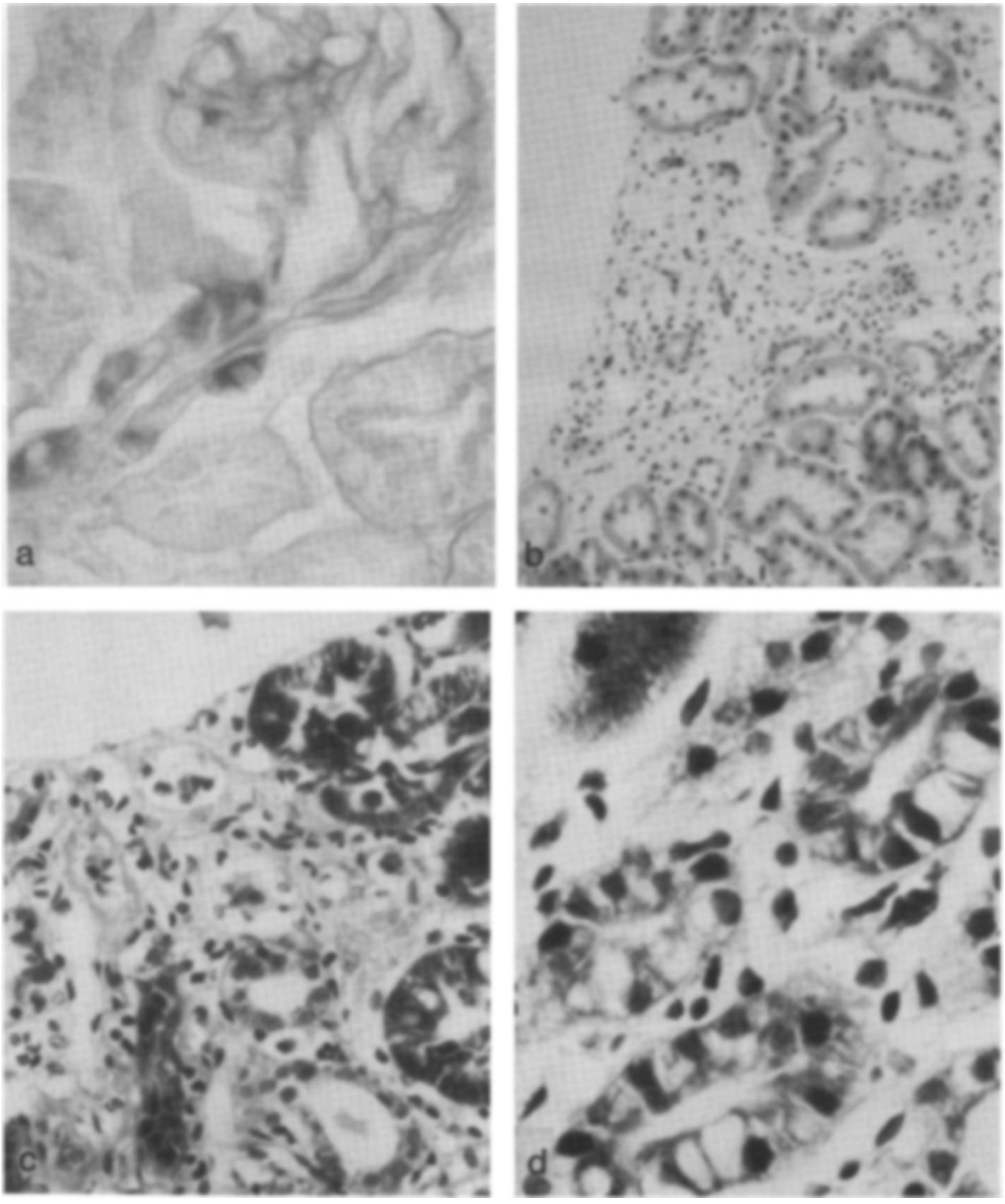

Fig. 1. *Histological staining of CsA-treated rat kidney tissue.* **A.** Periglomerular arterioles showing strong PAS staining- CsA, 7.5 mg/kg/day (× 200). **B.** Capsular notching and increased basophilia of underlying tubules- CsA, 12.5 mg/kg/day (H & E stains × 100). **C.** Section stained with Masson's trichrome showing subcapsular accumulation of collagen- CsA, 25 mg/kg/day (× 200). **D.** Proximal tubules showing isometric vacuolation, CsA 25 mg/kg/day (Masson's trichrome × 200).

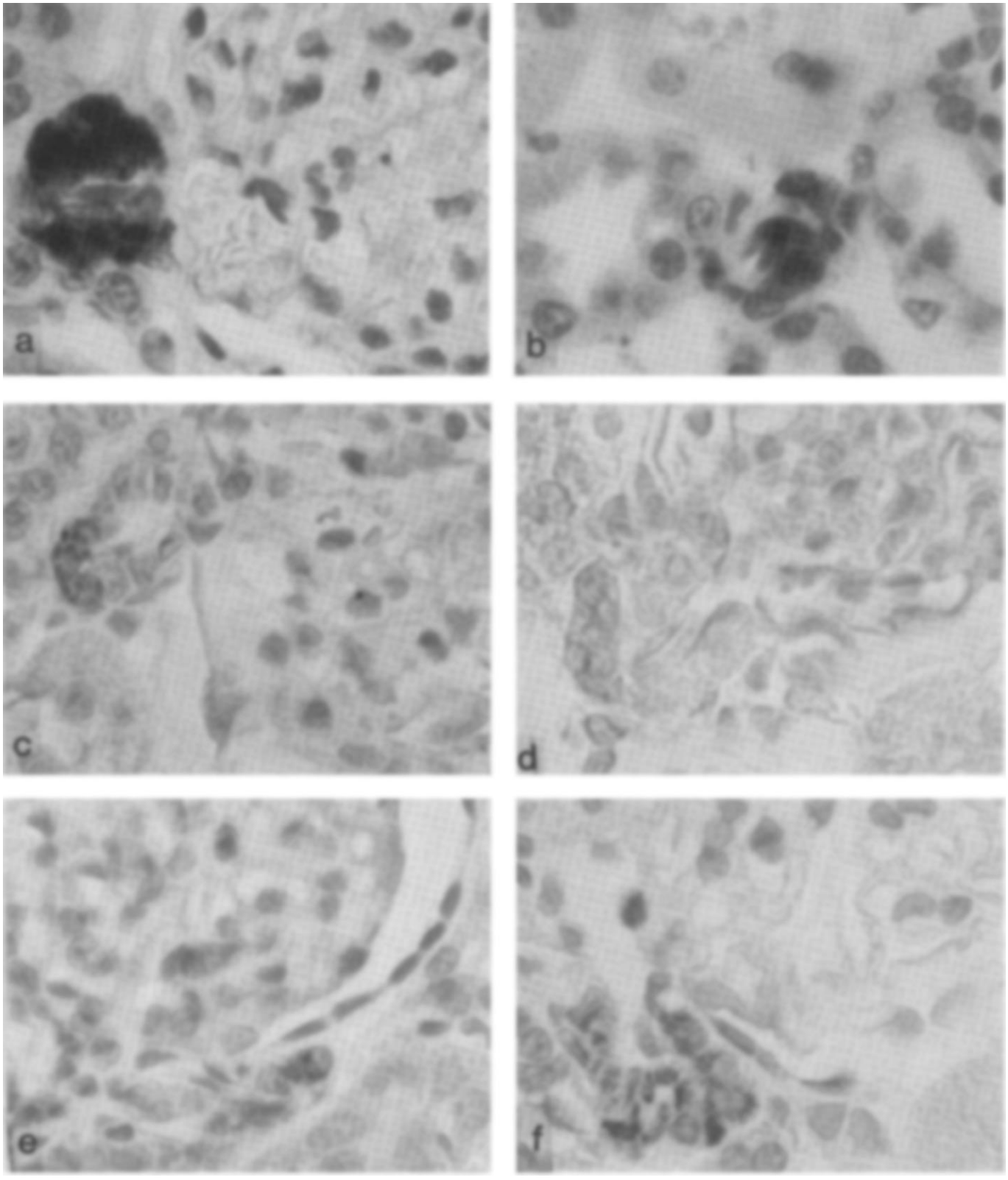

Fig. 2. *TGF-β immunohistochemistry.* **A.** CsA-treated (12.5 mg/kg/day) tissue showing intense staining of an hypertrophied afferent arteriole (× 400). **B.** CsA-treated (12.5 mg/kg/day) tissue showing strong staining of an interlobular arteriole (× 400). **C.** Control tissue showing weak staining confined to a few juxtaglomerular cells of an afferent arteriole (× 400). **D.** CsA-treated tissue (12.5 mg/kg/day) incubated with antibody preabsorbed with TGF-β1 showing no immunostaining of an hypertrophied afferent arteriole (× 400). **E.** Tissue from the right (non-ischemic) kidney of a rat subjected to unilateral renal ischemia demonstrating weak immunostaining of the afferent arteriole (× 400). **F.** Tissue from the left (ischemic) kidney of a rat subjected to unilateral ischemia showing moderate immunostaining (× 400).

Table 2. Body weight gain, blood pressure, serum levels of creatinine and potassium as well as creatinine clearance of rats subjected to unilateral ischaemia by partial ligation of the aorta for 2 weeks

	Body weight gain *g*	Serum creatinine *μmol/liter*	Serum potassium *mmol/liter*	Creatinine clearance rate *ml/min/100 g body wt*	Systolic blood pressure *mm Hg*
Sham ischemia ($N = 6$)	31 ± 5	30 ± 2	4.7 ± 0.4	0.53 ± 0.01	106 ± 9
Ischemia ($N = 6$)	24 ± 12	51 ± 5[a]	4.9 ± 0.6	0.38 ± 0.02	96 ± 12

Results are expressed as mean ± SEM.
[a] $P < 0.05$

Table 3. Intensity of staining of afferent and interlobular arterioles

	N	Weak	Moderate	Strong
Control	15	96	3	1
Pair-fed control	6	89	10	1
CsA, 5 mg/kg	3	78	19	3
CsA, 7.5 mg/kg	3	77	16	7
CsA, 10 mg/kg	3	39	37	24
CsA, 12.5 mg/kg	6	6	38	56
CsA, 25 mg/kg	12	9	19	79
Ischemia group	6			
Left kidney		34	51	15
Right kidney		71	27	2

Results are expressed as percentage of total number of arterioles which stained with TGF-β.

of TGF-β2 mRNA was noted. At the higher doses of CsA (12.5 to 25 mg/kg/day), there was significant CsA nephrotoxicity associated with a decline in renal function as well as vascular and tubular changes. These changes were also associated with hypercatabolism [37] as CsA-treated rats showed reduced weight gain compared to their respective controls.

Morphometric studies showed that although there was no increase in the frequency of glomerular profiles or α-SMA$_1$-stained arterioles in CsA-treated rats, there was a significant increase in the frequency of TGF-β-positive juxtaglomerular arterioles. In a previous study, we showed that CsA-treatment results in a 60% increase in the number of granules in juxtaglomerular cells [24]. The increase in stained complexes seen here is consistent with that observation and indicates a fourfold increase in the size of juxtaglomerular cell mass, either as a result of hyperplasia of pre-existing granulated cells or the metaplasic conversion of smooth muscle cells.

The EM studies show that TGF-β is localized within the secretion granules of juxtaglomerular cells both in CsA-treated and control olive oil-treated rats, although staining intensities varied from cell to cell. We have not undertaken a detailed analysis of the number of gold particles per granules as we consider this an unreliable form of quantification. Generally, there seemed to be somewhat fewer particles per secretion granule profile in the control olive oil-treated animals than in CsA-treated ones. However, the major difference between control and CsA-treated tissue was in the number of granulated juxtaglomerular cells per complex and the number of granules per cell.

Interactions between CsA and TGF-β have been described in other mammalian cell lines where CsA regulates cell growth via a TGF-β-dependent mechanism [38]. Cyclosporine has also been shown to stimulate the synthesis of TGF-β in human T lymphocytes [39]. In the kidney, other studies have shown that water deprivation [25] or potassium depletion [26] are potent stimuli for TGF-β expression within the juxtaglomerular apparatus. In this study, however, there was no evidence of hypokalemia nor increased TGF-β staining in pair-fed controls which had restricted amounts of water. The fact that control ischemic kidneys showed a comparable increase in juxtaglomerular arteriolar staining suggests that CsA-induced renal ischemia may be the determinant factor in the observed increase in arteriolar TGF-β. This is consistent with previous observations that renal artery stenosis in human neonates results in increased expression of juxtaglomerular TGF-β [27].

In this study, we have identified the presence of TGF-β within the secretion granules of the juxtaglomerular cells. We can only speculate, at this stage, as to the significance of this finding and its relevance to CsA-induced renal morphological changes. It is important to note, however, that this TGF-β is likely to be in an inactive form as this growth factor is normally activated upon secretion. Its localization within juxtaglomerular cells granules suggests that it is released in parallel with the vasoactive peptides produced by these cells. These observations also suggest that TGF-β may contribute to the CsA-induced hypertrophy and hypergranularity of the juxtaglomerular cells, whereas previously this role has been variously ascribed to renin, angiotensin II and most recently PDGF [24]. However, our finding is not inconsistent with these previous reports since interactions are known to take place between TGF-β, renin, angiotensin II and PDGF. For example, in juxtaglomerular cells in culture, TGF-β stimulates renin release [40], while in vascular smooth muscle cells angiotensin II has been shown to stimulate the release of TGF-β [41]. Furthermore, interactions between TGF-β and PDGF in vascular smooth muscle cells determine the nature of the growth response (hypertrophy or hyperplasia) of these cells to angiotensin II *in vitro* [42]. Such interactions between TGF-β and other growth promoters present within the juxtaglomerular cells may be instrumental in the hypertrophy of juxtaglomerular cells observed in CsA-treated rats.

TGF-β is also a well-known renal fibrogenic growth factor [43]. It has been shown to mediate the deposition of excess extracellular matrix proteins by mesangial cells in experimental [44] and human [45] glomerular diseases. Further, the transfection of rat glomeruli with the TGF-β1 gene leads to glomerulosclerosis [46]. Treatment of rats with experimental glomerulonephritis with a neutralizing anti-TGF-β1 antibody [47] or with a natural antagonist to TGF-β [48] confirmed a causal role for this growth factor in the pathogenesis of glomerulosclerosis. In view of the similarity between juxtaglomerular and mesangial cells [49], it is conceivable that TGF-β is also responsible for CsA-induced interstitial fibrosis. This may depend on paracrine interactions within the kidney.

In conclusion, our observation establishes an association between TGF-β and CsA-induced nephrotoxicity. It suggests that the observed changes in renal TGF-β may be linked to CsA-induced renal ischemia. However, confirmation of a causal link between TGF-β, renal ischemia and CsA-induced nephrotoxicity will depend on further studies, similar to those described above, and based on the manipulation of TGF-β.

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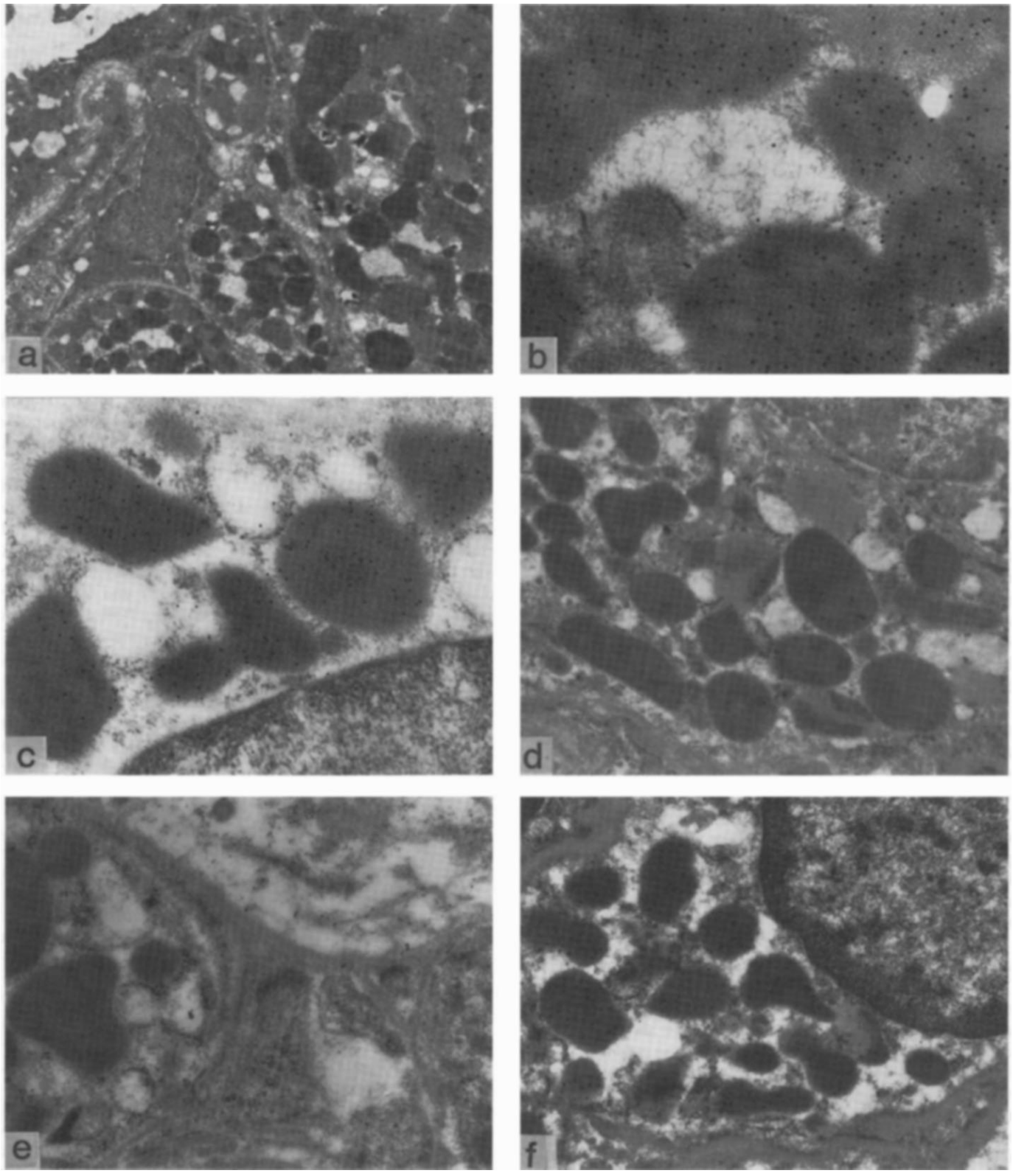

Fig. 3. *Immunocytochemistry of TGF-β using 10 nm colloidal gold probes-CsA, 12.5 mg/kg/day.* **A.** Survey electron micrograph showing hypertrophied juxtaglomerular cells with enlarged secretory granules labeled with colloidal gold (× 5,900). **B.** Portion of hypertrophied juxtaglomerular cells showing heavily labeled secretory granules. Note that the colloidal gold is confined to the granules, with no staining of the intervening cytoplasm (× 46,000). **C.** Control tissue showing lower density of labeling of the granules of the juxtaglomerular cell (× 37,000). **D.** CsA-treated tissue incubated with non-immune goat serum in place of TGF-β antibody showing no gold particles over the secretion granules of the juxtaglomerular cells (× 14,100). **E.** CsA-treated tissue incubated with goat anti-human collagen type IV antibody showing labeling of fibers in the extracellular matrix but no labeling of secretion granules (inappropriate antibody) (× 15,800). **F.** Tissue from left (ischemic) kidney of a rat subjected to unilateral renal ischemia. A pattern and intensity of labeling similar to that of CsA-treated tissue is evident (× 18,500).

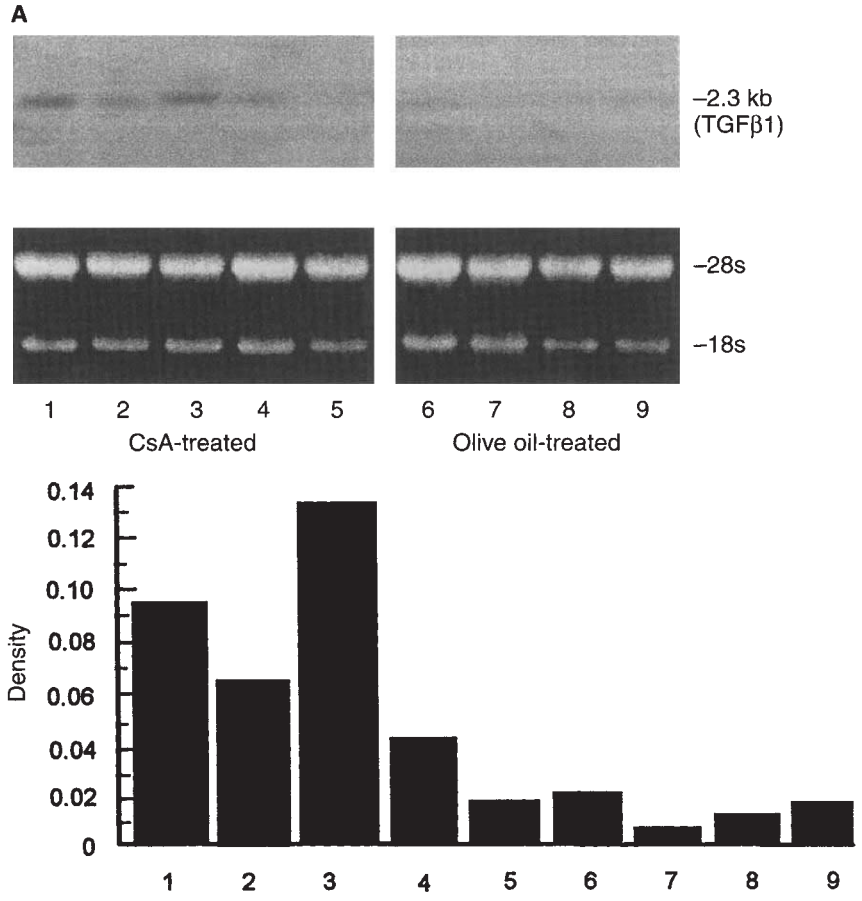

Fig. 4. *Northern blot analysis of rat kidney total RNA, hybridized with a ^{32}P labeled TGF-β1cDNA.* **A.** Left panel, CsA 12.5 mg/kg/day; right panel, olive oil control; upper panel, TGF-β1; lower panel, loading controls showing 28 s and 18 s RNA. **B.** Optical density of TGF-β1 mRNA of each animal.

Table 4. Frequency of glomerular profiles and of arterioles stained with α-smooth muscle actin and TGF-β per mm² cortex.

	N	Glomerular profiles per mm² cortex	Periglomerular arterioles α-SMA_1 per mm² cortex	Periglomerular arterioles TGF-β per mm² cortex	Interlobular arterioles TGF-β per mm² cortex
Control	15	5.65 ± 0.52	1.50 ± 0.09	0.21 ± 0.08	0.07 ± 0.06
Pair-fed control	6[d]	5.63 ± 0.29	1.59 ± 0.07	0.26 ± 0.04	0.05 ± 0.01
CsA, 5 mg/kg	3[e]	4.89 ± 0.43	1.48 ± 0.06	0.57 ± 0.02	0.19 ± 0.05
CsA, 7.5 mg/kg	3[e]	5.57 ± 0.38	1.52 ± 0.07	0.81 ± 0.07[b]	0.21 ± 0.08
CsA, 10 mg/kg	3[e]	5.74 ± 0.50	1.52 ± 0.10	1.18 ± 0.12[c]	0.34 ± 0.12
CsA, 12.5 mg/kg	6[e]	5.45 ± 0.70	1.51 ± 0.12	0.84 ± 0.15[b]	0.31 ± 0.02[a]
CsA, 25 mg/kg	12[f]	6.11 ± 0.21	1.46 ± 0.07	1.12 ± 0.10[c]	0.39 ± 0.08[b]
Ischemia group	6				
Left kidney		4.73 ± 0.31	1.20 ± 0.04	0.78 ± 0.09	0.19 ± 0.06
Right kidney		5.94 ± 0.73	1.43 ± 0.10	0.40 ± 0.06[a]	0.09 ± 0.01[a]

Results are expressed as mean ± SEM.
[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$
[d] Received an equal amount of food and water to that consumed by rats injected with 25 mg/kg/day CsA
[e] Values compared with control
[f] Values compared with pair-fed control

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References

1. KAHAN BD: Cyclosporine. *N Engl J Med* 321:1725–1738, 1989
2. PIRSCH JD, D'ALESSANDRO AM, KNECHTLE SJ, KALAYOGULU M, SOLLINGER HW, BELZER FO: Renal transplantation in cyclosporine era. *Transplant Proc* 25(Suppl 3):15–16, 1993
3. EUROPEAN MULTICENTRE TRIAL GROUP: Cyclosporine in cadaveric renal transplantation: 5-year follow-up of a multicentre trial. *Lancet* 2:506–507, 1987
4. MYERS BD: What is cyclosporine nephrotoxicity? *Transplant Proc* 21(Suppl 1):1430–1432, 1989
5. MYERS BD, SIBLEY R, NEWTON L, TOMLANOVICH SJ, BOSHKOS C, STENSON C, LUETSCHER JA, WHITNEY DJ, KRASNY D, COPLAN NS, PELROTH MG: The long-term course of cyclosporine nephropathy. *Kidney Int* 33:590–600, 1988

6. MYERS BD, NEWTON L: Cyclosporin induced chronic nephropathy: An obliterative microvascular renal injury. *JASN* 2(Suppl 1):454–452, 1991
7. MURRAY BM, PALLER MS, FERRIS TF: Effect of cyclosporine administration on renal hemodynamics in conscious rats. *Kidney Int* 28:767–774, 1985
8. SULLIVAN BA, HAK LJ, FINN WF: Cyclosporine nephrotoxicity: Studies in laboratory animals. *Transplant Proc* 17(Suppl 1):145–153, 1985
9. BARROS EJG, BOIM MA, AJZEN H, RAMOS OL, SCHOR N: Glomerular hemodynamics and hormonal participation on cyclosporine nephrotoxicity. *Kidney Int* 32:19–25, 1987
10. MIHATSCH MJ, THIEL G, SPICHTIN HP, OBERHOLZER M, BRUNNER FP, HARDER F, OLIVIERI V, BREMER R, RYFFEL B, STOCKLIN E, TORHORST J, GUDAT F, ZOLLINGER HU, LOERTSCHER R: Morphological findings in kidney transplants after treatment with cyclosporine. *Transplant Proc* 15(Suppl 1):2821–2835, 1983
11. SIEGL H, RYFFEL B, PETRIC R, SHOEMAKER P, MULLER P, DONATSCH P, MIHATSCH M: Cyclosporin, the renin-angiotensin-aldosterone system, and renal adverse reactions. *Transplant Proc* 15(Suppl 1):2719–2725, 1983
12. PERICO N, BENIGNI A, BOSCO E, ROSSINI S, ORISIO S, GHILARDI F, PICCINELLI A, REMUZZI G: Acute cyclosporine A nephrotoxicity in rats: Which role for renin-angiotensin system and glomerular prostaglandins? *Clin Nephrol* 25(Suppl 1):S83–S88, 1986
13. MURRAY BM, PALLER M: Beneficial effects of renal denervation and prazosin on GFR and renal blood flow after cyclosporine in rats. *Clin Nephrol* 25(Suppl 1):S37–S39, 1986
14. COFFMAN TM, CARR DR, YARGER WE, KLOTMAN PF: Evidence that prostaglandin and thromboxane production is stimulated in chronic cyclosporine nephrotoxicity. *Transplantation* 43:282–285, 1987
15. PERICO N, ROSSINI M, IMBERTO O, MALANCHINI B, CORNEJO RP, GASPARI F, BERTANI T, REMUZZI G: Thromboxane receptor blockade attenuates chronic cyclosporine nephrotoxicity and improves survival in rats. *JASN* 2:1398–1404, 1992
16. SMITH SR, CEECH EA, SCHAFFER V, MARTIN LL, RAKHIT A, DOUGLAS FL, KLOTMAN PE, COFFMAN TM: Effects of thromboxane synthase inhibition with CGS 13080 in human cyclosporine nephrotoxicity. *Kidney Int* 41:199–205, 1992
17. KON V, SUGIURA M, INAGAMI T, HARVIE BR, ICHIKAWA I, HOOVER RL: Role of endothelin in cyclosporine-induced glomerular dysfunction. *Kidney Int* 37:1487–1491, 1990
18. PERICO N, DADAN J, REMUZZI G: Endothelin mediates the renal vasoconstriction by cyclosporine in the rat. *JASN* 1:76–83, 1990
19. BLOOM ITM, BENTLET FR, GARRISON RN: Acute cyclosporine-induced renal vasoconstriction is mediated by endothelin-1. *Surgery* 114:480–488, 1993
20. GALLEGO MJ, LOPEZ FARRE A, RIESCO A, MONTON M, GRANDES SM, BARAT A, HERNANDO L, CASADO S, CARMELO CA: Blockade of endothelium-dependent responses in conscious rats by cyclosporin A: Effect of L-arginine. *Am J Physiol* 264:H708-H714, 1993
21. DENICOLA L, THOMSON SC, WEAD LM, BLANTZ RC, GABBAI FB: Cyclosporine A may decrease angiotensin II and diminish nitric oxide activity. *J Clin Invest* 92:1859–1865, 1993
22. SHEHATA M, RAFTERY AT, COPE GH, EL NAHAS AM: The role of nitric oxide in cyclosporin induced nephrotoxicity in the rat. (abstract) *JASN* 5:931, 1994
23. SHEHATA M: El Nahas AM, Barkworth E, Cope GH, Raftery AT: Increased platelet-derived growth factor in the kidneys of cyclosporin-treated rats. *Kidney Int* 46:726–732, 1994
24. SHEHATA M: El Nahas AM, Barkworth E, Cope GH, Raftery AT: Localisation of PDGF-BB in the juxtaglomerular cells of cyclosporin-treated rats. *Exp Nephrol* 3:173–179, 1995
25. HORIKOSHI S, MCCUNE BK, RAY PE, KOPP JB, SPORN MB, KLOTMAN PE: Water deprivation stimulates transforming growth factor-β2 accumulation in the juxtaglomerular apparatus of mouse kidney. *J Clin Invest* 88:2117–2122, 1990
26. RAY PE, MCCUNE BK, GOMEZ AR, HORIKOSHI S, KOPP JB, KLOTMAN PE: Renal vascular induction of TGF-β2 and renin by potassium depletion. *Kidney Int* 44:1006–1013, 1993
27. RAY PE, MCCUNE BK, GOMEZ RA, RULEY EJ, KLOTMAN PE: Expression of transforming growth factor β3 in hypertrophic juxtaglomerular apparatus. *N Engl J Med* 330:68–69, 1994
28. CANTIN M, ARAUJO-NASCIMENTO M-D-F, BENCHIMOL S, DESMORMEAUX Y: Metaplasia of smooth muscle cells into juxtaglomerular cells in the juxtaglomerular apparatus, arteries and arterioles of the ischaemic (endocrine) kidney. *Am J Pathol* 87:581–602, 1977
29. PRICE A, GRAY TA, BENNETT K, CRUISE M: Comparison of three immunoassay methods for the measurement of cyclosporine. *Clin Chem Enzym Comms* 4:163–174, 1994
30. SKALLI O, ROPRAZ P, TRZECIAK A, BENZONANA G, GILLESSEN D, GABBIANI G: A monoclonal antibody against α-smooth muscle actin: A new probe for smooth muscle differentiation. *J Cell Biol* 102:1726–1737, 1986
31. ROTH J: Post-embedding cytochemistry with gold-labelled reagents: A review. *J Microsc* 143:125–137, 1986
32. DERYNCK R, JARRET JA, CHEN EY, GOEDDER DV: The murine transforming growth factor-β precursor. *J Biol Chem* 261:4377–4379, 1986
33. MILLAN AF, DENHEZ F, KONDAIAH P, AKHURST RJ: Embryonic gene expression patterns of TGF-β1, β2 suggest different development functions in vivo. *Development* 111:131–134, 1991
34. MILLER D, LEE A, PELTOW RW, CHEN EY, MOSES HY, DERYNCK R: Murine transforming growth factor β2 cDNA sequence and expression in adult tissue and embyros. *Mol Endocrinol* 3:1108–1114, 1989
35. SAIKI PK, GEZFAND DH, STOFFEZ S, SCHARF SJ, HIHUCHI R, HORN GT, MULLS KB, ERLICH HA: Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–491, 1988
36. CHURCH GM, GILBERT W: Genomic sequencing. *Proc Natl Acad Sci USA* 81:1991–1995, 1984
37. DIEPRINK H, STARKLINT H, LEYSSAC PP: Nephrotoxicity of cyclosporine - An animal model: Study of the nephrotoxic effect of cyclosporine on overall renal and tubular function in conscious rats. *Transplant Proc* 15(Suppl 1):2736–2741, 1983
38. KHANNA A, LI B, STENZEL KH: Regulation of new DNA synthesis in mammalian cells by cyclosporine. Demonstration of a transforming growth factor beta-dependent mechanism of inhibition of cell growth. *Transplantation* 57:577–582, 1994
39. LI B, SEHAJPAL PK, KHANNA A, VLASSARA H, CERAMI A, STENZEL KH, SUTHANTHIRAN M: Differential regulation of transfroming growth factor beta and interleukin 2 genes in human T cells: Demonstration by usage of novel competitor DNA constructs in the quantitative polymerase chain reaction. *J Exp Med* 174:1259–1262, 1991
40. ANTONIPILLAI I, HOANG LE T, SOCENEATU L, HORTON R: Transforming growth factor-β is a renin secretagogue at picomolar concentrations. *Am J Physiol* 265:F537–F541, 1993
41. STOUFFER GA, OWENS GK: Angiotensin II-induced mitogenesis of spontaneously hypertensive rat-derived cultured smooth muscle cells is dependent on autocrine production of transforming growth factor-β. *Circ Res* 70:820–828, 1992
42. GIBBONS GH, PRATT RE, DZAU VJ: Vascular smooth muscle cell hypertrophy vs. hyperplasia, autocrine transforming growth factor-β1 expression determines growth response to angiotensin II. *J Clin Invest* 90:456–461, 1992
43. BORDER WA, NOBLE NA: Transforming growth factor-β in glomerular injury. *Exp Nephrol* 2:13–18, 1994
44. OKUDA S, LANGUINO LR, RUOSLAHTI E, BORDER WA: Elevated expression of transforming growth factor-β and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix. *J Clin Invest* 86:453–462, 1990
45. YOSHIOKA K, TAKEMURA T, MURAKAMI K, OKUDA M, HINO S, MYASATO H, MAKI S: Transforming growth factor-β and mRNA in glomeruli in normal and diseased human kidneys. *Lab Invest* 68:154–163, 1993
46. ISAKA Y, FUJIWARA Y, UEDA N, KANEDA Y, KAMADA T, IMAI E: Glomerulosclerosis induced by *in vivo* transfection of TGF-β and PDGF gene into the rat kidney. *J Clin Invest* 92:2597–2601, 1993
47. BORDER WA, OKUDA S, LANGUINO LR, RUOSLAHTI E: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β1. *Nature* 346:371–374, 1990
48. BORDER WA, NOBLE NA, YAMAMOTO T, HARPER JR, YAMAGUCHI Y, PIERSCHBACHER MD, RUOSLAHTI E: Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. *Nature* 360:361–364, 1992
49. DUNIHUE FW, BOLDOSSER WG: Observations on the similarity of mesangial to juxtaglomerular cells. *Lab Invest* 12:1228–1240, 1963